Structure, Biosynthesis, and Localization of Dipeptidyl Aminopeptidase B, an Integral Membrane Glycoprotein of the Yeast Vacuole

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Abstract. We have characterized the structure, biogenesis, and localization of dipeptidyl aminopeptidase B (DPAP B), a membrane protein of the yeast vacuole. An antibody specific for DPAP B recognizes a 120-kD glycoprotein in yeast that behaves like an integral membrane protein in that it is not removed from membranes by high pH Na_2CO_3 treatment. Inspection of the deduced amino acid sequence of DPAP B reveals a hydrophobic domain near the NH_2 terminus that could potentially span a lipid bilayer. The in vitro enzymatic activity and apparent molecular weight of DPAP B are unaffected by the allelic state of *PEP4*, a gene essential for the proteolytic activation of a number of soluble vacuolar hydrolases. DPAP B is synthesized as a glycosylated precursor that is converted to the mature 120-kD species by carbohydrate addition. The precursor form of DPAP B accumulates in *sec* mutants (Novick, P., C. Field, and R. Schekman. 1980. *Cell.* 21:205–215) that are blocked at the ER (*sec18*) or Golgi apparatus (*sec7*), but not at secretory vesicles (*sec1*). Immunolocalization of DPAP B in wild-type or *sec1* mutant cells shows that the protein resides in the vacuolar membrane. However, it is present in non-vacuolar compartments in *sec18* and *sec7* cells, confirming that the delivery of DPAP B is blocked in these mutants. Interestingly, DPAP B appears to stain the nuclear envelope in a *sec18* mutant, which is consistent with the accumulation of DPAP B in the ER membrane at the restrictive temperature. These results suggest that soluble and membrane-bound vacuolar proteins use the same stages of the secretory pathway for their transport.

THE vacuole of the yeast *Saccharomyces cerevisiae* is considered to be the equivalent of the lysosome of mammalian cells because it contains a large number of hydrolytic enzymes and has an acidic pH (reviewed in Rothman and Stevens, 1988). The biosynthesis and sorting of soluble vacuolar hydrolases, such as carboxypeptidase Y (CPY)[1] and proteinase A, have been examined. The localization of soluble proteins to the vacuole requires the early stages of the secretory pathway; i.e., ER and Golgi apparatus (Stevens et al., 1982). In addition, localization determinants on CPY and proteinase A, as well as genes necessary for the efficient sorting of these proteins, have been identified (Valls et al., 1987; Johnson et al., 1987; Klionsky et al., 1988; Rothman and Stevens, 1986; Bankaitis et al., 1986; Robinson et al., 1988; Rothman et al., 1989).

Much less is known about the sorting of membrane proteins to the vacuole. Studies on mammalian cells indicate that soluble and membrane-bound lysosomal proteins are sorted by distinct mechanisms (von Figura and Hasilik, 1986). Several lysosomal membrane proteins have been identified (Chen et al., 1985; Lewis et al., 1985; Barriocanal et al., 1986; Lippincott-Schwartz and Fambrough, 1986; Waheed et al., 1988), and of those examined, none possess the mannose-6-phosphate moiety required for sorting soluble lysosomal proteins (von Figura and Hasilik, 1986). Furthermore, several membrane proteins are found at normal levels in the lysosomes of I-cell fibroblasts, which missort soluble lysosomal hydrolases to the cell surface.

Several activities associated with the vacuolar membrane of yeast have been characterized (Rothman and Stevens, 1988), including transport systems for Ca^{2+} (Ohsumi and Anraku, 1981) and basic amino acids (Ohsumi and Anraku, 1983), a protein-translocating ATPase (Uchida et al., 1985; Kane et al., 1989), an α-mannosidase (Opheim, 1978), and dipeptidyl aminopeptidase (DPAP) B (Suarez Rendueles et al., 1981; Julius et al., 1983; Bordallo et al., 1984). Nothing is known about the transport or posttranslational modifications for any of the vacuolar membrane proteins. Analysis of the localization of α-mannosidase and DPAP B in yeast mutants that missort soluble vacuolar proteins suggested that most of these mutants do not missort membrane-bound vacuolar proteins (Bankaitis et al., 1986; Rothman and

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1. *Abbreviations used in this paper*: CPY, carboxypeptidase Y; DPAP, dipeptidyl aminopeptidase; endo F, endoglycosidase F; N-linked, asparagine-linked.

Stevens, 1986; Robinson et al., 1988; Rothman and Stevens, unpublished results). Thus, soluble and membrane-bound proteins appear to be directed to the vacuole by distinct mechanisms.

As a first step in the study of vacuolar membrane protein sorting and transport, we have characterized the structure, biogenesis, and localization of DPAP B. We show that DPAP B is an integral membrane protein that, like soluble vacuolar proteins, uses the early stages of the secretory pathway for its transport to the vacuole.

Materials and Methods

Strains, Growth Conditions, and Materials

The genotypes of the *Escherichia coli* and *S. cerevisiae* strains used in these studies are listed in Table I. Yeast cultures were grown in minimal medium supplemented with the appropriate nutrients as previously described (Stevens et al., 1986).

Restriction endonucleases and other enzymes used for cloning and sequencing were from New England Biolabs (Beverly, MA), Bethesda Research Laboratories (Gaithersburg, MD), Pharmacia Fine Chemicals (Piscataway, NJ), or Boehringer Mannheim Biochemicals (Indianapolis, IN). ^{125}I-protein A was from Amersham Corp. (Arlington Heights, IL) and carrier-free ^{35}S-H_2SO_4 was from ICN Biomedicals Inc. (Irvine, CA). IgG Sorb was from The Enzyme Center (Boston, MA), endoglycosidase F (endo F) was from Boehringer Mannheim Biochemicals, and FITC-conjugated sheep anti-rabbit IgG was from Cappel Laboratories (Malvern, PA). All other reagents used in these experiments were from Sigma Chemical Co. (St. Louis, MO).

Plasmid Constructions, Recombinant DNA Methodology, and DNA Sequence Analysis

Restriction endonuclease and Bal 31 exonuclease digestions and ligations were performed as recommended by the suppliers. Plasmid purification, agarose gel electrophoresis, and DNA-mediated transformation of *E. coli* were performed according to standard procedures (Maniatis et al., 1982). Plasmids were introduced into yeast cells by the lithium acetate transformation method (Ito et al., 1983). *E. coli* strain MC1061 was used for all plasmid manipulations and JM103 was used for M13 phage work.

DNA sequencing was performed by the chain termination method of Sanger et al. (1977) using M13mp18 and mp19 vectors (Yanisch-Perron et al., 1985). In one case, sequencing was primed using a synthetic oligonucleotide corresponding to nucleotides 441-459 of DAP2 (see Fig. 2), prepared at the University of Oregon Biotechnology Laboratory on a DNA synthesizer (model 380B; Applied Biosystems, Inc., Foster City, CA) as described (Ito et al., 1982). DNA and amino acid sequence analyses were performed using the sequence analysis software package (version 4) of the University of Wisconsin Genetics Computer Group.

Plasmid pRG1 was constructed by inserting the 4.7-kb Bam HI–Pst I (blunt-ended by treating with T4 DNA polymerase) *DAP2* fragment (Fig. 1), isolated from the original *DAP2* plasmid YEp13-GS13-4 (Julius et al., 1983), into the Bam HI–Sma I sites of pUC12. Plasmid pGP3, a multicopy 2-μm plasmid containing the *DAP2* gene, was constructed by inserting the 4.7-kb Bam HI–Pst I (blunt-ended) fragment from pRG1 into the Bam HI–Pvu II sites of YEp24.

A chromosomal *DAP2* deletion was constructed by transplacing the genomic copy with the cloned gene into which the *LEU2* gene was inserted in place of 1.3 kb of the *DAP2* coding region (Rothstein, 1983; Fig. 1). Plasmid pRG1 was digested with BstE II and Kpn I, treated with T4 DNA polymerase to make blunt ends, and ligated with a 2.1-kb Hpa I fragment containing the *LEU2* gene. The resulting plasmid (pGP2) was cut with Bam HI and Pst I and used to transform the yeast strain JHRY20-2C (leu2-3, leu2-112). Stable *LEU+* transformants were purified and screened for the lack of thermolabile dipeptidyl aminopeptidase activity, assayed as described previously (Julius et al., 1983).

Plasmid pCJR6, a multicopy 2-μm yeast vector containing the DPAP B coding region fused to the *GAL1* promoter, was constructed by inserting a 400-bp Eco RI–Hind III *GAL1* promoter fragment (Johnston and Davis, 1984) into the Eco RI and Hind III sites of pSEY8, and subsequently subcloning into the Hind III site of this plasmid (pCJR5) the 2.9 kbp Hind III fragment of *DAP2* (Fig. 1), resulting in the fusion of the *GAL1* promoter at position −90 of *DAP2*.

DPAP B antigen for production of antiserum was made in *E. coli* by fusing a portion of the *DAP2* coding region into the *E. coli* expression vector pHSe5, which contains the T4 phage lysozyme gene under the control of the TAC promoter (Muchmore et al., 1988; kindly provided by R. Dahlquist, University of Oregon). Plasmid pRG1 was digested with Stu I, treated briefly with Bal 31 exonuclease, and further digested with Hind III. Fragments of ~1,650 bp were cloned into the Sna BI–Hind III sites of pHSe5,

Table I. Strains Used in This Study

Yeast strain	Genotype	Source or reference
X2180-1AL1	*MATa gal2 lys2 mal mel*	Derived from X2180
HMSF176	*MATa sec18 gal2*	Novick et al., 1980
SF294-2B	*MATa sec7 gal2*	Novick and Schekman, 1983
HMSF1	*MATa sec1 gal2*	Novick et al., 1980
SEY5186	*MATa sec18 ura3-52 leu2-3 leu2-112*	Emr et al., 1984
CJRY21-3BΔ1	*MATa sec18 ura3-52 leu2-3 leu2-112 met14 ade1 ade2-1 trp1 dap2-Δ2::LEU2 GAL+*	This study
CJRY22-6BΔ1	*MATa sec1 ura3-52 leu2-3 leu2-112 met14 ade1 ade2-1 dap2-Δ2::LEU2 GAL+*	This study
CJRY23-2AΔ1	*MATa sec7 ura3-52 leu2-3 leu2-112 met14 ade1 his3 dap2-Δ2::LEU2 GAL+*	This study
JHRY20-1AΔ1	*MATa ura3-52 leu2-3 leu2-112 his3-Δ200 lys2-801 pep4-3 can1 ste13-Δ1::LEU2*	Rothman, 1988
JHRY20-2C	*MATa ura3-52 leu2-3 leu2-112 his3-Δ200*	Rothman, 1988
JHRY20-2CΔ2	*MATa ura3-52 leu2-3 leu2-112 his3-Δ200 pep4-Δ2::LEU2*	Derived from JHRY20-2C
JHRY20-2CΔ3	*MATa ura3-52 leu2-3 leu2-112 his3-Δ200 dap2-Δ2::LEU2*	Derived from JHRY20-2C
E. coli		
MC1061	*F⁻ hsdR hsdM+ araD139 (araABOIC-leu)7679 (lac)X74 galU galK rpsL*	Casadaban and Cohen, 1980
JM103	*F' Δ(lac pro) thi strA endA sbcB15 supE/F' traD36 proAB+ lacIq lacZΔM15*	Messing, 1983

resulting in the production of a hybrid protein consisting of the NH_2-terminal seven residues of T4 phage lysozyme fused to the COOH-terminal half of DPAP B. Transformants were screened for an isopropyl β-D-thiogalactopyranoside- (IPTG) inducible protein species by SDS-PAGE (Laemmli, 1970) of total *E. coli* cell extracts. A plasmid was isolated (pRG2) which produced a large amount (~5% of total cell protein) of a 55-kD species upon induction with isopropyl β-D-thiogalactopyranoside.

Production of DPAP B Antigen and Generation of DPAP B Antiserum

E. coli cells containing plasmid pRG2 were grown in LB broth plus ampicillin to saturation, then diluted 1:100 in 250 ml of fresh medium, and grown for 1 h at 37°C. Isopropyl β-D-thiogalactopyranoside was added to 1 mM, and incubation was continued for 3 h before collecting the cells by centrifugation. The cells were lysed and the fusion protein was isolated from the insoluble fraction essentially as previously described (Kleid et al., 1981). An SDS-PAGE gel slice containing the fusion protein was resuspended in SDS elution buffer (1% SDS, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 100 mM NaCl) in a homogenizer (Dounce; Wheaton Instruments Div., Millville, NJ), incubated for 2 h at 37°C, and centrifuged at 12,000 *g* for 10 min. The supernatant was saved, and the pellet was resuspended in 5 ml of elution buffer and treated as above. The supernatants were combined, and the protein was precipitated by adding 4 vol of acetone, incubating for 30 min at −80°C, and centrifuging at 15,000 *g* for 10 min.

A DPAP B-specific antibody was prepared by injecting DPAP B antigen into New Zealand White Rabbits (250 μg DPAP B antigen per rabbit per injection) essentially as described (Vaitukaitis, 1981). These antibodies were affinity purified as previously described (Stevens et al., 1982). Antiserum to phosphoglycerate kinase was prepared as described (Rothman et al., 1986).

Alkaline Sodium Carbonate Fractionation

Yeast cells containing plasmid pGP3 were grown in minimal medium at 30°C to mid log phase. 12 OD_{600} U of cells (1.2×10^8 cells) were spheroplasted as described previously (Stevens et al., 1986), lysed in 50 mM sodium phosphate, pH 7.5, 2 mM EDTA, and 0.5 mM PMSF, and diluted 100-fold in ice-cold 100 mM sodium carbonate, pH 11.5. An aliquot representing the whole extract fraction was removed, and after 30 min on ice the lysate was centrifuged for 3 h at 100,000 *g*. The supernatant and whole extract fractions were neutralized with 1 N acetic acid, then precipitated in 5% TCA on ice. Precipitated protein was pelleted, washed twice in diethylether, dried, resuspended in sample buffer, and heated at 70°C until dissolved. The pellet fraction was homogenized in 50 mM Tris-HCl, pH 6.8, and solubilized by addition of SDS to a final concentration of 4%. The sample was incubated at 60°C until complete solubilization had occurred; i.e., the suspension became completely transparent. Sample buffer (without SDS) was added, and the samples were subjected to SDS-PAGE on an 8% polyacrylamide gel. Electroblotting of the gel onto nitrocellulose was carried out according to Burnette (1981) using a Trans-blot Apparatus from Bio-Rad Laboratories (Richmond, CA). The transferred proteins were detected by probing the nitrocellulose with antibodies to the appropriate antigens and subsequent labeling with 0.3 μCi of ^{125}I-protein A. The dried nitrocellulose was exposed to film (XAR-5; Eastman Kodak Co., Rochester, NY) at −80°C.

Radiolabeling, Immunoprecipitation, and Endo F Treatment

Yeast cells were grown in minimal media plus 50 μM Na_2SO_4 (Stevens et al., 1986) to mid log phase, spun down in a clinical centrifuge, washed once and resuspended in sulfate-free media, and incubated at the appropriate labeling temperature for 15–30 min at 8 OD_{600} U/ml. ^{35}S-H_2SO_4 was added (0.5–1.0 mCi) and cells were labeled and then chased with 10 mM Na_2SO_4 (except for the experiments in Fig. 5 *B*, which were chased with 10 mM Na_2SO_4, 1 mM cysteine, 1 mM methionine, and 0.1 mg/ml cycloheximide). 2 OD_{600} U of cells were then sedimented, converted to spheroplasts, and lysed in 0.1 ml of 1% SDS by heating for 3 min at 100°C. 0.9 ml of immunoprecipitation buffer (Stevens et al., 1986) was added, followed by 0.1 ml of IgG Sorb (prepared as suggested by the manufacturer). The mixture was centrifuged 15 min in a microfuge (model 235B; Fisher Scientific Co., Pittsburgh, PA) and the supernatant was treated with 2 μl of affinity purified DPAP B antibody and incubated 60 min on ice. The immune complexes were sedimented with IgG Sorb and washed as before (Stevens et al., 1982), and the final IgG Sorb cell pellet was resuspended in 0.1 ml of 1% SDS and heated 3 min at 100°C. 0.9 ml of immunoprecipitation buffer and 0.1 ml of IgG Sorb was added, and the immunoprecipitation protocol was repeated. Sample buffer was added to the final IgG Sorb pellet and the samples were heated for 3 min at 100°C and centrifuged. Supernatant fractions were transferred to new tubes and aliquots (normalized for the amount of ^{35}S-H_2SO_4 incorporated) were analyzed by SDS-PAGE and fluorography as described previously (Stevens et al., 1986).

Endo F treatment (Elder and Alexander, 1982) was carried out on immunoprecipitated samples of DPAP B essentially as described (Stevens et al., 1986). A time course of endo F treatment was performed by adding 300 mU of endo F to DPAP B immunoprecipitated from SEY5186 cells (containing plasmid pGP3) and withdrawing aliquots at the times indicated in Fig. 6.

Immunofluorescence Microscopy

Yeast cells containing a disruption of the chromosomal *DAP2* locus and transformed with the plasmid pCJR6 were grown in minimal media plus 2% raffinose to an OD_{600} of 1.0, and galactose was added directly to the cultures to a final concentration of 3%. The cultures were then incubated at either 34°C (*SEC*$^+$, *sec18*, and *sec1*) or 38°C (*SEC*$^+$ and *sec7*) for 2 h and the cultures were put on ice. The cells were fixed with formaldehyde, spheroplasted, and prepared for immunofluorescence as described (Kilmartin and Adams, 1984; Adams and Pringle, 1984; Wittenberg et al., 1987). In some cases, fixed spheroplasts were treated with SDS by resuspending in 1 ml of 1.2 M sorbitol/PBS (prepared as described by Adams and Pringle, 1984) plus 5% SDS, mixed gently, centrifuged at 8,000 *g*, and washed twice in 1.2 M sorbitol. FITC-conjugated sheep anti-rabbit IgG second antibody was used for staining DPAP B antibody. Nuclei were stained with 4',6-diamidino-2-phenyl-indole (DAPI) as described (Kilmartin and Adams, 1984).

Cells were viewed and photographed using a Zeiss Axioplan Photomicroscope (equipped for epifluorescence at excitation wavelengths appropriate for DAPI and FITC fluorescence) and film and developer (TMAX-400; Eastman Kodak Co.). Photomicrographs are shown at a magnification of 1,000.

Results

The DAP2 Gene Encodes DPAP B

Yeast cells contain two membrane-associated DPAP activities that differ in their subcellular locations and thermal stabilities (Suarez Rendueles et al., 1981; Julius et al., 1983). DPAP B is a thermolabile enzyme associated with the vacuolar membrane (Bordallo et al., 1984). DPAP A, the *STE13* gene product, is a nonvacuolar, heat-stable enzyme that participates in the proteolytic maturation of the mating pheromone α-factor (Fuller et al., 1988). The *STE13* gene was cloned by complementation of the mating defect of a *MATα*

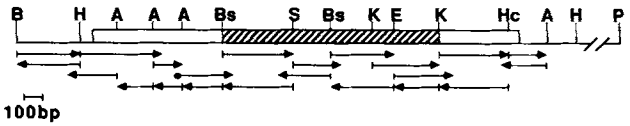

Figure 1. Restriction map and nucleotide sequencing strategy of the *DAP2* gene. A linear map of the *DAP2* gene is depicted, with the horizontal arrows indicating direction and extent of sequence determination. The arrow originating in a solid circle corresponds to sequence determined from DNA synthesis primed by a synthetic oligonucleotide. The boxed region denotes the open reading frame, with the predicted initiating methionine codon at the left end. The hatched region shows the portion of *DAP2* coding sequence replaced by *LEU2* sequences in the *dap2-Δ2::LEU2* disruption (see Materials and Methods). Restriction sites that are relevant to the sequence analysis are indicated: *B*, Bam HI; *H*, Hind III; *A*, Acc I; *Bs*, BstE II; *S*, Stu I; *K*, Kpn I; *E*, Eco RI; *Hc*, Hinc II; *P*, Pst I.

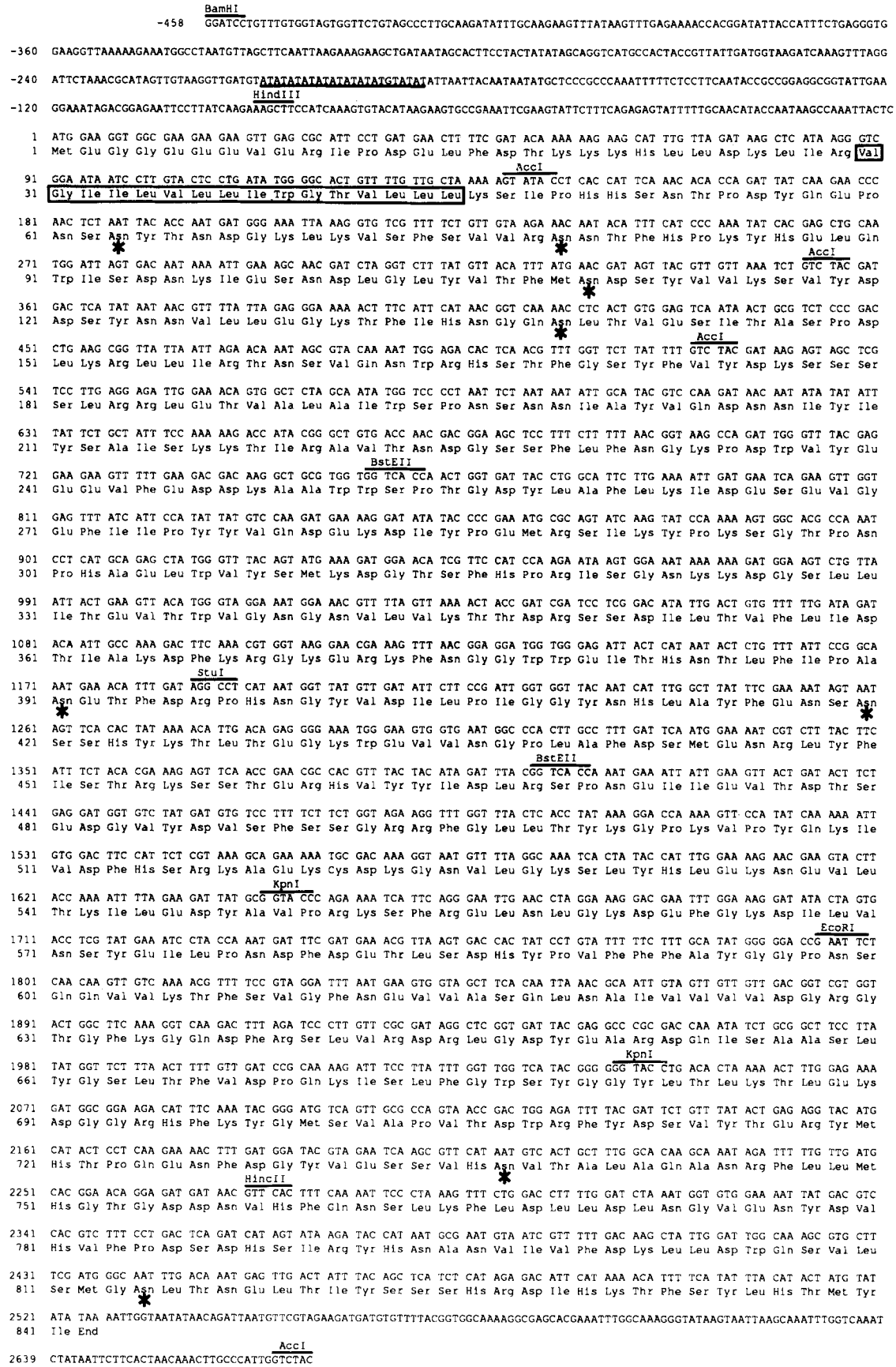

Figure 2. Nucleotide and corresponding amino acid sequence of the *DAP2* gene. Nucleotide residues are numbered in the 5′- to -3′ direction, with the *A* of the predicted ATG initiating methionine given the number 1. The restriction sites used in sequencing are indicated. The TATA-like element is underlined, the amino acids comprising the putative membrane-spanning domain are boxed, and the potential sites of N-linked glycosylation are marked by asterisks.

ste13 mutant (Sprague, G., and Ira Herskowitz, unpublished results; Julius et al., 1983). Another gene, called *STE13'* (also called *DPP2*), was cloned by its ability to complement the α-factor maturation defect of a *ste13* mutant when expressed from a high copy number plasmid (Sprague, G., and I. Herskowitz, unpublished results; Julius et al., 1983). The gene dosage of *DPP2* had a direct effect on the specific activity of DPAP B in extracts of yeast cells (Julius et al., 1983; Sprague, G., unpublished results). Strains transformed with a multicopy plasmid containing *DPP2* on a 4.7-kb Bam HI–Pst I fragment (Fig. 1) showed a 10–20-fold increase in DPAP B activity, whereas strains carrying a disrupted genomic copy of *DPP2* (*dpp2-Δ2::LEU2*, see Materials and Methods; see Fig. 4) showed no measurable DPAP B activity in assays of whole cell extracts (data not shown). Also, a *dpp2-Δ2::LEU2/DPP2* diploid strain had ~50% of the DPAP B activity of a *DPP2/DPP2* diploid (data not shown).

Mutants deficient in DPAP B activity have been previously described (Suarez Rendueles and Wolf, 1987). These mutants, including one with an exceptionally thermolabile DPAP B activity, harbored mutations that fell into a single complementation group, called *DAP2*, and failed to complement the *dpp2-Δ2* mutation. Thus, *DPP2* and *DAP2* define the same locus, which encodes DPAP B. Henceforth, *DAP2* will denote the gene encoding DPAP B. No phenotype other than the lack of DPAP B was observed in strains disrupted at the *DAP2* locus (Sprague, G., unpublished results; data not shown), consistent with the results of Suarez Rendueles and Wolf (1987).

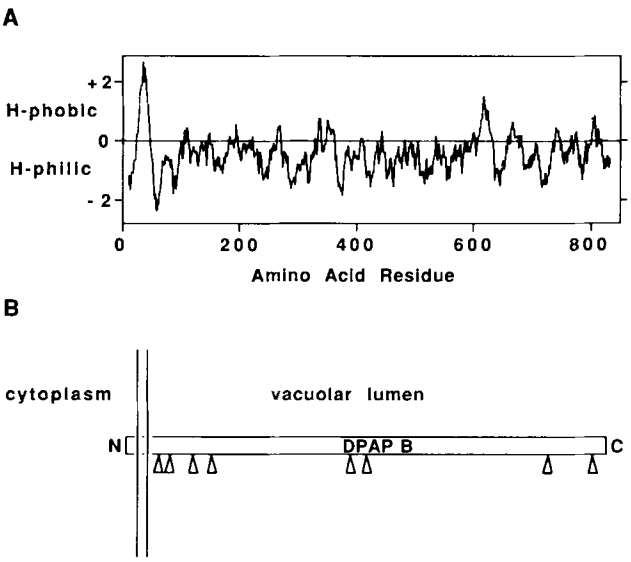

Figure 3. Hydrophobicity analysis of the *DAP2* gene product. (*A*) The mean hydropathic index of successive stretches of 19 amino acid residues is plotted vs. the amino acid number of the middle residue of each stretch. Regions with high positive values are potential membrane spanning domains. (*B*) Model of the membrane topology of DPAP B predicted from the deduced amino acid sequence. The NH_2- and COOH-terminal ends of the protein are indicated, and the sites for N-linked glycosylation are marked by triangles.

Primary Structure of DPAP B Deduced from the DAP2 Sequence

We deduced the primary structure of DPAP B by sequencing *DAP2*. Fig. 1 shows the restriction map of *DAP2* and the sequencing strategy for the 3,135-bp Bam HI–Acc I fragment. The complete nucleotide and deduced amino acid sequences are presented in Fig. 2. 459 bp of 5' and 153 bp of 3' flanking sequences are included in addition to the 2,523 bp of coding sequence.

Translation of DPAP B is predicted to initiate at the methionine codon numbered 1 (Fig. 2). The sequenced region contains a long open reading frame coding for a protein of 841 amino acids, with eight canonical sites for asparagine-linked (N-linked) glycosylation. A 24-bp segment located from −210 to −187 relative to the putative translation initiating codon may function as a reiterated TATA-like box for initiation of transcription. An identical sequence is found in a similar location upstream of the yeast *STE3* gene (Hagen et al., 1986). The predicted molecular mass from the deduced amino acid sequence is 96,429 D, which is similar to the apparent molecular mass of DPAP B after removal of N-linked carbohydrate (see below).

Hydropathy analysis of the amino acid sequence (Kyte and Doolittle, 1982; Fig. 3 *A*) reveals a single hydrophobic domain, beginning 30 residues from the NH_2 terminus, of sufficient length and hydrophobicity to span a lipid bilayer (Adams and Rose, 1985; Davis and Model, 1985). Thus, the predicted topology of DPAP B is that of a type II (or group B; Fig. 3 *B*) integral membrane protein (Garoff, 1985; Singer et al., 1987), with an NH_2-terminal cytoplasmic domain and a lumenal COOH terminus. The hydrophobic domain presumably functions as both an ER-targeting signal and a membrane anchor, as is true of other type II integral membrane proteins, such as the neuraminidase of influenza virus (Bos et al., 1984) and the transferrin receptor (Zerial et al., 1986). Further support for this disposition of DPAP B in the membrane is the observation that DPAP B is a glycoprotein (see below), and that all sites for N-linked glycosylation are included in the COOH-terminal domain, which therefore must be the lumenal domain.

No significant sequence similarity has been found between DPAP B and any other vacuolar or lysosomal protein for which sequence information is available. However, strong homology has been found between DPAP B and DPAP A, the product of the *STE13* gene (Flanagan, C., and J. Thorner, personal communication; see below).

DPAP B Is a 120-kD Integral Membrane Protein

To facilitate the biochemical characterization of DPAP B, a DPAP B–specific antibody was prepared (see Materials and Methods). Lanes *1* and *2* of Fig. 4 show that the antibody is specific for the *DAP2* gene product in yeast. Isogenic *DAP2* and *dap2-Δ2* cells were labeled with ^{35}S-H_2SO_4 for 30 min and chased for 30 min in the presence of 10 mM Na_2SO_4, and immunoprecipitations were performed from cell extracts. Two species were precipitated from the *DAP2* strain (Fig. 4, lane *2*): a major species of ~120 kD and a lesser amount of a 110-kD form. Both of these bands were absent from the *dap2-Δ2* strain (lane *1*), indicating that they represent the products of the *DAP2* locus.

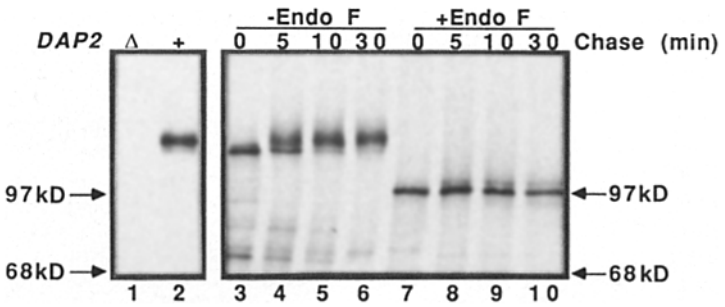

Figure 4. Immunoprecipitations of DPAP B. (Lanes *1* and *2*) JHRY20-2CΔ3 (Δ) and JHRY20-2C (+) cells were ^{35}S labeled for 30 min and chased 30 min in the presence of Na_2SO_4. (Lanes *3–10*) JHRY20-2C cells were pulse labeled for 5 min and chased in unlabeled Na_2SO_4, cysteine, methionine, and cycloheximide for the times indicated. Labeled cells were collected, converted to spheroplasts, lysed in 1% SDS, and immunoprecipitated with DPAP B antibody. Immunoprecipitates were analyzed on 6% polyacrylamide SDS gels without (*−Endo F*) or with (*+Endo F*) earlier treatment with endo F.

The deduced amino acid sequence predicts that DPAP B is an integral membrane protein. To test this prediction biochemically, yeast spheroplasts were treated with 100 mM sodium carbonate, pH 11.5, and centrifuged at 100,000 *g*. Under these conditions, only intrinsic membrane proteins pellet with membranes, whereas soluble and peripheral membrane proteins are recovered in the supernatant (Steck and Yu, 1973; Fujiki et al., 1982). Fig. 5 shows western blot analysis of the total, pellet, and supernatant fractions using DPAP B–specific and phosphoglycerate kinase– (PGK) specific antibodies. The DPAP B antigen was found in the membrane fraction, whereas the soluble protein PGK did not sediment with membranes. These data confirm that DPAP B is an integral membrane protein.

DPAP B Is Synthesized as a Glycosylated Precursor

The soluble vacuolar proteins analyzed to date, such as CPY, are synthesized as glycoproteins (Rothman and Stevens, 1988). CPY first appears as a glycosylated precursor which receives further glycosyl residues in the Golgi apparatus before being proteolytically cleaved to the mature form upon delivery to the vacuole (Stevens et al., 1982). To follow the biosynthesis of DPAP B, a culture of *DAP2* cells was pulse labeled for 5 min in ^{35}S-H_2SO_4 and chased for 0, 5, 10, and 30 min (Fig. 4, lanes *3–6*). DPAP B initially appeared as two distinct bands of 110 and 113 kD (lane *3*) which chased to the broad 120-kD species seen in lane *2*. A small amount of the 110-kD form remained after a 30-min chase (lanes *2* and *6*). Longer times of up to 60 min were required to convert this species to the fully mature 120-kD form (data not shown).

Treatment of the immunoprecipitated samples with endo F (Fig. 4, lanes *7–10*), which removes N-linked glycosyl residues, showed that the only detectable difference in the species seen in lanes *3–6* is in the amount of N-linked carbohydrate present on DPAP B. At each chase time point, labeled DPAP B migrated as a 96-kD species after endo F treatment, which is very close to the molecular mass predicted from the deduced amino acid sequence. A small amount of a 99-kD species was also detected in lanes *8–10*; this appears to correspond to one residual carbohydrate chain remaining on DPAP B since it disappears upon longer incubation with endo F (see Fig. 6). Thus, DPAP B is modified in a manner similar to soluble vacuolar proteins, such as CPY, in that its carbohydrate moieties undergo only modest extension in the Golgi apparatus, and do not receive the extensive outer chain mannose residues found on secreted proteins such as invertase (Esmon et al., 1981; Runge, 1988).

DPAP B Receives Variable Numbers of N-linked Glycosyl Residues

The appearance of two distinct DPAP B species at the earliest times in its biogenesis is similar to that seen with invertase, which is initially synthesized as several discrete species that differ in the number of N-linked glycosyl chains added per protein molecule (Esmon et al., 1981). To determine how many of the eight potential sites for N-linked glycosylation are modified on DPAP B, limited endo F digestion was per-

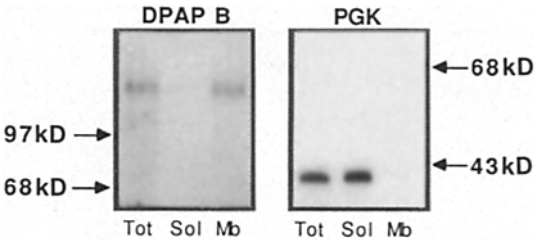

Figure 5. DPAP B is an integral membrane protein. JHRY20-1AΔ1 cells containing the *DAP2* gene on a multicopy plasmid were converted to spheroplasts, lysed, and diluted into 100 mM sodium carbonate, pH 11.5. A portion of the total extract (*Tot*) was saved, and the rest was separated into membrane (*Mb*) and soluble (*Sol*) fractions by centrifugation at 100,000 *g* for 3 h. The three fractions were subjected to SDS-PAGE, and the gel was analyzed by immunoblotting with DPAP B and phosphoglycerate kinase antibodies and subsequent incubation with ^{125}I–protein A. An autoradiogram of the immunoblots is shown, with the positions of the protein molecular mass standards indicated.

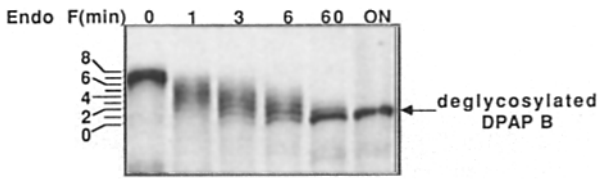

Figure 6. Time course of endo F digestion. DPAP B was immunoprecipitated from CJRY21-3BΔ1 (*sec18*) cells containing *DAP2* on a multicopy 2-μm plasmid after ^{35}S labeling for 30 min. Samples were digested with endo F for the times indicated (*ON*, overnight). The different glycosylated species are marked, with the corresponding number of glycosyl residues shown.

formed on the precursor, which was immunoprecipitated from a *sec18* mutant at 34°C (see below). Fig. 6 suggests that the precursor forms seen in lane *3* of Fig. 4 correspond to DPAP B with six and seven glycosyl residues added, although forms with five and eight oligosaccharide chains can also be seen (Fig. 6, 0-min time point). Therefore, DPAP B can be modified at all eight or as few as five sites, but the majority of molecules receives six or seven glycosyl chains.

DPAP B Is Not Affected by the Allelic State of PEP4

The *PEP4* gene function is necessary for the activation of a number of vacuolar hydrolases (Jones et al., 1982). Mutations at this locus result in the failure to proteolytically process the large molecular mass, inactive forms of soluble vacuolar proteins such as CPY (Rothman and Stevens, 1988). Suarez Rendueles et al. (1981) showed that total DPAP (DPAP A and DPAP B) activity was similar in a wild-type strain and a *pep4-3* mutant. To examine further the effect of a *pep4* mutation on DPAP B, enzyme assays and immunoprecipitations of DPAP B were performed using isogenic *PEP4* and *pep4-Δ2* strains. No significant effect on the activity of DPAP B was detected in the *pep4-Δ2* strain as compared to the *PEP4* strain (data not shown), confirming that DPAP B is not synthesized as an inactive precursor. This result is consistent with the observation that DPAP B which had accumulated in the ER of *sec18* cells (see below) was enzymatically active (data not shown). Fig. 7 shows that DPAP B immunoprecipitated from isogenic *PEP4* and *pep4-Δ2* strains comigrates before and after digestion with endo F, although a minor breakdown product, not seen in the *pep4-Δ2* strain, was detected in the *PEP4* strain after endo F treatment. These data argue that the biogenesis of DPAP B is independent of the allelic state of the *PEP4* gene.

Early-blocked sec Mutants Accumulate the Precursor Form of DPAP B

The temperature-sensitive *sec* mutations define sequential steps in the secretory pathway in yeast, since cells carrying these mutations are blocked for protein secretion at distinct stages (Novick et al., 1980; Novick et al., 1981). The localization of CPY to the vacuole is prevented in *sec* mutants that are blocked at either the ER or Golgi apparatus (*sec18* and *sec7*, respectively; Stevens et al., 1982), and the protein accumulates as an inactive precursor that lacks Golgi-mediated carbohydrate modifications. *sec1* cells, which at 37°C are blocked at a late secretory vesicle stage of the pathway, transport CPY to the vacuole at all temperatures. Thus, secretory and soluble vacuolar proteins diverge after the *sec7*-defined block and before the *sec1*-blocked stage. To determine if the maturation of DPAP B was affected in these mutants, DPAP

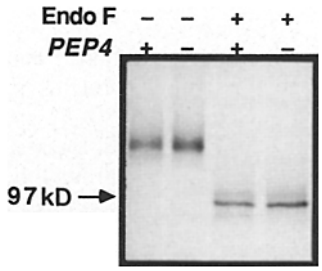

Figure 7. Effect of a *pep4* mutation on DPAP B. Strains JHRY20-2C and JHRY20-2CΔ2 were ^{35}S labeled for 15 min and chased for 30 min at 30°C. Labeled cells were collected, and DPAP B was immunoprecipitated and endo F treated as before.

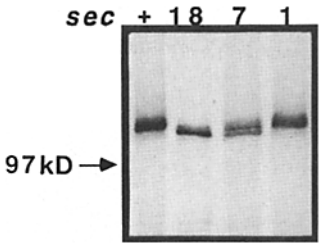

Figure 8. Effect of *sec* mutations on DPAP B. Strains were ^{35}S labeled at their nonpermissive temperatures (X2180, HMSF176, and HMSF1, 34°C; SF294-2B, 38°C) for 15 min, chased for 30 min in the presence of Na_2SO_4 at the same temperature, and DPAP B was immunoprecipitated as before.

B was immunoprecipitated after labeling the cells at the nonpermissive temperature of 34°C (Salminen and Novick, 1987), except for the *sec7* mutant, whose restrictive temperature is 38°C (data not shown). Wild-type cells contained the 120-kD mature form of DPAP B at either 34°C (Fig. 8) or 38°C (data not shown). *sec1* cells also contained mature DPAP B, whereas *sec18* cells accumulated the DPAP B precursor. Thus, the precursor form seen after a short labeling period (Fig. 4, lane *3*) is the ER-form of DPAP B. The *sec7* strain accumulated a mixture of core-glycosylated and mature forms of DPAP B. This suggests that the block in transport is at an early stage within the Golgi apparatus such that DPAP B is only partially processed by the mannosyl transferases (Runge, 1988). This phenotype is similar to that seen with invertase, which accumulates as a heterogeneous population of core- and hyper-glycosylated forms in *sec7* cells at the restrictive temperature (Esmon et al., 1981).

The accumulation of the DPAP B precursor in *sec18* and *sec7* cells was thermoreversible: after labeling cells at the restrictive temperature, shifting the cultures to 23°C, and chasing for 3 h in the absence of new protein synthesis, DPAP B was completely converted to the mature 120-kD form (data not shown). This result, which was also observed with CPY accumulated in *sec18* and *sec7* cells (Stevens et al., 1982), suggests that the accumulated DPAP B precursor resides early in the pathway and is able to transit the Golgi apparatus upon shifting to the permissive temperature.

Early-blocked sec Mutants Accumulate DPAP B in Nonvacuolar Compartments

To determine whether the accumulation of the DPAP B precursor in *sec18* and *sec7* mutants was correlated with a failure to deliver the protein to the vacuole, DPAP B was localized by indirect immunofluorescence microscopy. For these studies, DPAP B synthesis was placed under the control of the inducible *GAL1* promoter (Johnston and Davis, 1984), allowing analysis of nascent DPAP B synthesis after shifting to the nonpermissive temperature. A multicopy plasmid containing the *GAL1-DAP2* gene fusion was transformed into wild-type and *sec* mutant strains which had been disrupted at the chromosomal *DAP2* locus. Transformed strains were then shifted to the restrictive temperatures in galactose-containing media and incubated for 2 h. This procedure typically resulted in a 4–10-fold induction of DPAP B activity relative to a strain containing a single genomic copy of *DAP2* (data not shown). No DPAP B activity or antigen was detected when these strains were grown in the absence of galactose (data not shown). The cells were fixed in formaldehyde, spheroplasted, and incubated with affinity-purified DPAP B

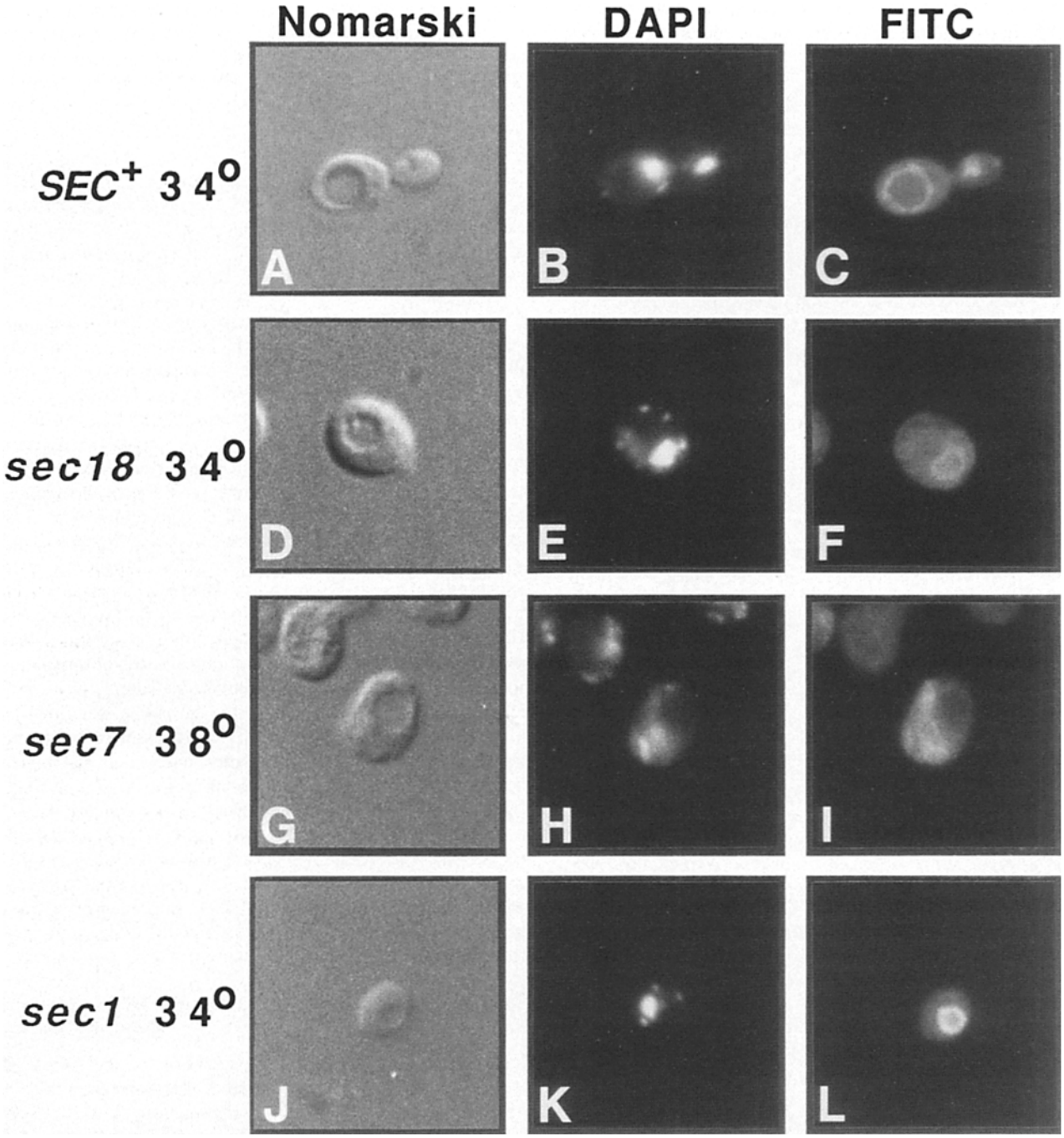

Figure 9. DPAP B localization by immunofluorescence microscopy. Strains JHRY20-1A (*SEC*$^+$), CJRY21-3BΔ1 (*sec18*), CJRY23-2AΔ1 (*sec7*), and CJRY22-6BΔ1 (*sec1*), each containing the GAL1-DAP2 gene fusion on a multicopy plasmid, were shifted to their respective nonpermissive temperatures and treated for induction of the *GAL1* promoter for 2 h as described in Materials and Methods. The cells were fixed, converted to spheroplasts, and stained with DAPI and affinity-purified DPAP B antibody. Cells were viewed by Nomarski differential interference optics (*A*, *D*, *G*, and *J*), and under DAPI (*B*, *E*, *H*, and *K*) and FITC (*C*, *F*, *I*, and *L*) excitation wavelengths.

antibody, followed by FITC-conjugated sheep anti-goat antibody (Fig. 9). Nomarski optics revealed the position of the vacuole, and the nucleus was localized by DAPI staining. Wild-type cells grown at either 34°C (Fig. 9, *A–C*) or 38°C (data not shown) exhibited fluorescent labeling of DPAP B around the perimeter of the vacuole, consistent with the fact that DPAP B is a vacuolar membrane protein. A similar labeling pattern was seen in *sec1* cells (Fig. 9, *J–L*), indicating that the *sec1* block is later in the secretory pathway than the point at which sorting of vacuolar membrane proteins occurs. The staining pattern seen in *sec18* (Fig. 9, *D–F*) and *sec7* (Fig. 9, *G–I*) cells was distinctly nonvacuolar. Interestingly, the *sec18* strain showed DPAP B staining around the nucleus, apparently in the nuclear envelope, which probably reflects the fact that the nuclear envelope and the ER are continuous membranes. These data indicate that transport of

DPAP B to the vacuole is prevented in *sec* mutants that are blocked in protein transport early in the secretory pathway.

Discussion

DPAP B is the first protein of the yeast vacuolar membrane whose structure and biosynthesis has been characterized. Of the vacuolar membrane-associated activities that have been described in yeast, only the proton-translocating ATPase (Uchida et al., 1985; Kane et al., 1989) and α-mannosidase (Yoshihisa et al., 1988) have been extensively characterized biochemically. The proton-translocating ATPase is a multimeric enzyme consisting of both integral and peripheral membrane proteins (Kane et al., 1989). α-Mannosidase, which is frequently used as a marker for the vacuolar membrane, is efficiently removed from membranes by treatment with high-pH sodium carbonate, suggesting that it is peripherally associated with the vacuolar membrane (Yoshihisa et al., 1988). Thus, DPAP B appears to be a more appropriate marker for intrinsic vacuolar membrane proteins.

In a previous biochemical study, DPAP B was reported to be a 40-kD protein (Garcia Alvarez et al., 1985). In the present work we have found that DPAP B is a 120-kD glycoprotein, that this species is absent from a strain with a disruption at *DAP2*, and that the *DAP2* sequence encodes a protein predicted to have a molecular mass of 96,429 D, which corresponds closely to that actually observed for deglycosylated DPAP B. The 40-kD species seen previously may have been a contaminating protein or a proteolytic fragment of DPAP B.

As is the case for the soluble vacuolar protein CPY (Stevens et al., 1982), the delivery of DPAP B to the vacuole is blocked in *sec* mutants that affect the early stages of the secretory pathway. *sec18* (ER-blocked) and *sec7* (Golgi-blocked) cells accumulate DPAP B in nonvacuolar compartments at the restrictive temperature. However, *sec1* cells, which are blocked at a late secretory vesicle stage, transport DPAP B to the vacuole at all temperatures, indicating that DPAP B is transported directly from the Golgi apparatus to the vacuole. This rules out models in which vacuolar membrane proteins are first delivered to the plasma membrane, and suggests that, in yeast, soluble and membrane-bound proteins traverse the same compartments of the secretory pathway en route to the vacuole (Stevens et al., 1982), as is the case for the delivery of proteins to the lysosome in mammalian cells (Griffiths et al., 1988).

The activity and apparent molecular mass of DPAP B are unaffected by a null mutation at the *PEP4* locus. DPAP B is active at all stages of its transport and does not appear to undergo proteolytic cleavage upon delivery to the vacuole. In this respect, DPAP B differs from the soluble vacuolar hydrolases studied thus far, which are synthesized as zymogens and are activated in a *PEP4*-dependent fashion (Rothman and Stevens, 1988). Interestingly, the other vacuolar membrane activities that have been examined, i.e., α-mannosidase (Jones et al., 1982) and the proton-translocating ATPase (Yamashiro, C., and T. Stevens, unpublished results), are also *PEP4* independent.

Overproduction of the soluble vacuolar proteins CPY and proteinase A results in their secretion, presumably due to the saturation of a component of the sorting apparatus (Stevens et al., 1986; Rothman et al., 1986). This observation provided a means of isolating mutations that disrupt the sorting process, both linked and unlinked to the structural genes of the soluble vacuolar proteins (Valls et al., 1987; Rothman and Stevens, 1986; Bankaitis et al., 1986; Robinson et al., 1988; Rothman et al., 1989). 20-fold overproduction of DPAP B does not result in increased cell surface activity of this enzyme (our unpublished results). However, at this level of expression DPAP B does suppress the defect in α-factor maturation of a *MATα ste13* mutant (Sprague, G., and I. Herskowitz, unpublished observation; Julius et al., 1983). A reasonable interpretation of this result is that DPAP B is mislocalized into the late secretory pathway when overproduced, allowing it to encounter and process the α-factor precursor to the mature form. Based on this assumption, mutations have been isolated that result in secretion of mature α-factor in a *ste13* background. These mutants do not cause DPAP B to be overproduced, and may define genes that encode components of the sorting apparatus for vacuolar membrane proteins (Pohlig, G., and T. Stevens, unpublished results).

The sorting signal(s) that directs membrane proteins to the vacuole has yet to be defined. The sorting information could reside in any of three distinct domains; i.e., the cytoplasmic, membrane, and/or lumenal domains. Moreover, each of these domains has been shown to be important for proper intracellular targeting of particular membrane proteins. The lumenal domains of the influenza virus hemagglutinin and vesicular stomatitis virus G protein have been implicated as important for the polarized secretion of these proteins to the apical and basolateral membranes, respectively (Roth et al., 1987; McQueen et al., 1987). Retention of the coronavirus E1 glycoprotein in the Golgi apparatus requires a specific transmembrane region (Machamer and Rose, 1987), and an intact cytoplasmic tail is necessary for endocytosis of the LDL and transferrin receptors (Davis et al., 1986; Rothenberger et al., 1987). Preliminary experiments have shown that the cytoplasmic tail and transmembrane region of DPAP B contain vacuolar sorting information; i.e., fusion of the NH_2-terminal 47 residues of DPAP B to the NH_2 terminus of the cytoplasmic form of invertase results in the delivery of this hybrid protein to the vacuolar membrane (Roberts, C., and T. Stevens, unpublished observation). This approach has been previously used to map the sorting domains of CPY (Johnson et al., 1987) and proteinase A (Klionsky et al.,

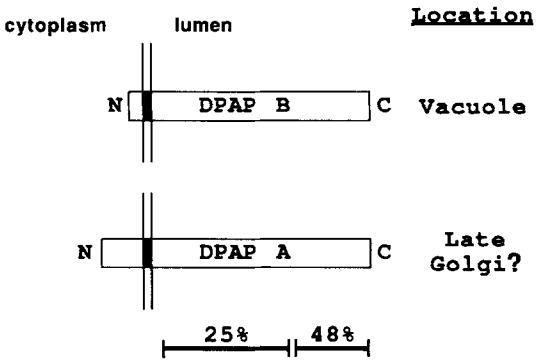

Figure 10. Comparison of DPAP B and DPAP A. Models for the disposition of the two proteins in their respective membranes are shown, with their subcellular locations listed at right. The NH_2 and COOH termini of the proteins are indicated, as are the regions of extensive amino acid identity, with the percentage of identical amino acids given.

1988). Further experiments are aimed at the identification of the specific amino acid residues in this region that are necessary for targeting DPAP B to the vacuole.

The DNA sequence of the *STE13* gene, which encodes DPAP A (Julius et al., 1983), has recently been determined (Flanagan, C., and J. Thorner, personal communication). The deduced amino acid sequence predicts a protein of 931 amino acids with striking similarities to DPAP B (Fig. 10). Both proteins are predicted to have type II membrane topologies, with short cytoplasmic tails and ~800 amino acid lumenal domains. The lumenal domains of these proteins are very similar; in particular, the COOH-terminal 240 amino acids are 48% identical, presumably reflecting the similar enzymatic functions of these proteins in vitro and in vivo (Julius et al., 1983). However, the cytoplasmic and transmembrane domains are unrelated, possibly reflecting the different subcellular addresses of DPAP A and DPAP B. This sorting signal hypothesis can be tested by exchanging these segments of the two proteins, an approach that has been useful in studying the sorting of membrane proteins in polarized cells (Roth et al., 1987; McQueen et al., 1987).

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